Virology **264,** 159–166 (1999)
Article ID viro.1999.9980, available online at http://www.idealibrary.com on IDEAL®

The A20 Protein Interacts with the Epstein–Barr Virus Latent Membrane Protein 1 (LMP1) and Alters the LMP1/TRAF1/TRADD Complex

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Received May 25, 1999; returned to author for revision June 28, 1999; accepted August 24, 1999

The Epstein–Barr virus (EBV) latent membrane protein 1 (LMP1) interacts with the tumor necrosis factor receptor (TNFR)-associated factor (TRAF) molecules, which are important for LMP1-mediated signaling. Two domains of LMP1 can independently activate NF-kB, carboxyl-terminal activating region 1 (CTAR1) and CTAR2. The activation of NF-kB by CTAR1 occurs through direct interaction of LMP1 with the TRAF molecules, whereas CTAR2 interacts with the TNFR-associated death domain protein (TRADD) to activate NF-kB and the c-Jun N-terminal kinase (JNK). A20, which is induced by LMP1 through NF-kB, can block NF-kB activation from both domains of LMP1 and inhibit JNK activation from CTAR2. A20 also has been shown to associate with TRAF1 and TRAF2. In this study, an interaction between LMP1 and A20 was detected that was increased by TRAF2 overexpression. A20 did not affect the association of TRAF1 with TRAF2 but did displace TRAF1 from the LMP1 complex. The interaction of LMP1 and TRADD was decreased in the presence of A20, and the LMP1-A20 association was decreased by TRADD, suggesting that A20 and TRADD both interact with LMP1 and may compete for binding. These data indicate that A20 alters the interactions between LMP1 and the TRAF molecules and TRADD, affecting the activation of NF-kB, JNK, and perhaps other TRAF-mediated signaling events.

INTRODUCTION

Epstein–Barr virus (EBV) is associated with a variety of lymphoid and epithelial malignancies including Burkitt's lymphoma (BL), posttransplant lymphoma (PTL), and nasopharyngeal carcinoma (NPC) (zurHausen *et al.,* 1970; Desgranges *et al.,* 1975; Hanto *et al.,* 1982; Geser *et al.,* 1983; Raab-Traub and Flynn, 1986; Raab-Traub *et al.,* 1987; Katz *et al.,* 1989). Preinvasive lesions related to NPC contain clonal EBV DNA and express latent membrane protein (LMP1) in all abnormal cells, suggesting that EBV and LMP1 are important in the development of NPC (Pathmanathan *et al.,* 1995). LMP1 is the only EBV-encoded protein able to transform established rodent fibroblasts and is also essential for transformation of primary B lymphocytes (Wang *et al.,* 1985; Kaye *et al.,* 1993). LMP1 induces profound changes in cellular gene expression due partly to activation of the NF-kB transcription factor. In lymphocytes, LMP1 induces the expression of cell-surface markers such as CD21, CD23, CD40, and CD44, cell adhesion molecules ICAM1, LFA1, and LFA3, and the anti-apoptotic molecules bcl-2 and A20 (D. Wang *et al.,* 1988; F. Wang *et al.,* 1990; Henderson *et al.,* 1991; Laherty *et al.,* 1992; Peng and Lundgren, 1993; Huen *et al.,* 1995). In epithelial cells, LMP1 induces expression of the epidermal growth factor receptor (EGFR) as well as A20 (Miller *et al.,* 1995). LMP1 and A20 both protect epithelial cells from p53-mediated apoptosis induced by serum withdrawal (Fries *et al.,* 1996).

LMP1 initiates signaling events in part through association with the tumor necrosis factor receptor-associated factors (TRAFs) (Mosialos *et al.,* 1995; Devergne *et al.,* 1996). In addition to LMP1, the TRAFs interact with the cytoplasmic domains of the tumor necrosis factor receptor (TNFR) superfamily members. The ability of LMP1 to transform primary B lymphocytes *in vitro* is dependent on interaction with the TRAFs and clustering of LMP1 in the plasma membrane (D. Wang *et al.,* 1988a,b; Izumi *et al.,* 1997). The TRAF binding site has been defined further as amino acids 204–208 of LMP1, with a consensus motif of Pro-X-Gln-X-Thr (with X representing any amino acid) that also is present in CD40 and CD30 (Hu *et al.,* 1994; Devergne *et al.,* 1996; Gedrich *et al.,* 1996) The TRAF interaction domain, which lies within carboxyl-terminal activating region 1 (CTAR1), is one of the two distinct domains in LMP1 that are able to activate the NF-kB transcription factor (Huen *et al.,* 1995; Mitchell and Sugden, 1995). The second domain of LMP1 that can activate NF-kB (CTAR2) is located within the last 35 amino acids and can interact with the adaptor protein TNFR-associated death domain protein (TRADD) (Izumi and Kieff, 1997). Although direct interactions have not been detected between CTAR2 and the TRAF molecules, NF-kB activation by this domain can be decreased by a dominant negative TRAF2 protein as well as the TRAF inhib-

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0042-6822/99 $30.00

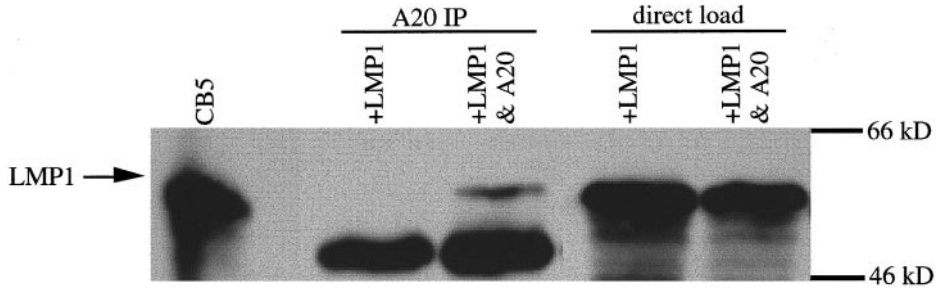

FIG. 1. Interaction of EBV LMP1 with A20. The H1299-p53 cell line was transfected with either LMP1 alone or LMP1 and c-myc-tagged A20. Immunoprecipitation with c-myc monoclonal antibody agarose conjugate was performed to immunoprecipitate c-myc-tagged A20, followed by immunoblotting with an LMP1 monoclonal antibody. Direct loads of 1% of the samples were taken before immunoprecipitation to verify LMP1 protein expression. CB5 is an EBV-infected lymphoblastoid cell line included as a positive control for LMP1.

itor TANK (Kaye *et al.,* 1996). The CTAR2 domain also activates c-Jun N-terminal kinase (JNK) (Eliopoulos *et al.,* 1999). A20 blocks the activation of JNK and NF-kB by LMP1 through a previously undetermined mechanism (Eliopoulos *et al.,* 1999).

Expression of the EBV LMP1 protein in epithelial cells protected cells from p53-mediated apoptosis in the H1299 cell line containing a temperature-sensitive form of p53 (Fries *et al.,* 1996). The antiapoptotic protein A20 was induced by LMP1 in this cell line and expression of A20 alone without LMP1 also protected from apoptosis mediated by p53. Induction of A20 by LMP1 therefore may underlie the ability of LMP1 to protect EBV-infected epithelial cells from p53-mediated apoptosis. However, little is known about how A20 functions. A20 interacts with TRAF1, and this interaction is strengthened by expression of TRAF2, suggesting that A20 associates with TRAF1/TRAF2 heterodimers (Song *et al.,* 1996). TRAF2 is able to mediate NF-kB activation by the TNFR type I (TNFRI), TNFRII, EBV LMP1, and CD40 (Rothe *et al.,* 1995; Hsu *et al.,* 1996). LMP1 also can interact with the TRAF molecules, with the molar ratios of TRAF1, TRAF2, and TRAF3 present in the LMP1-TRAF complex affecting signaling pathways (Mosialos *et al.,* 1995; Devergne *et al.,* 1996; Miller *et al.,* 1997, 1998). In this study, potential interactions between LMP1 and A20 were investigated. These data indicate that A20 associates with the LMP1-TRAF and LMP1-TRADD complexes, displacing TRAF1 and TRADD. The alteration of TRAF complex formation by A20 is likely responsible for its inhibition of LMP1 activation of JNK and NF-kB.

RESULTS

EBV LMP1 is able to interact with A20 in the H1299-p53 cell line

A yeast two-hybrid screen using the LMP1 carboxyl-terminus identified an interaction with TRAF3 (Mosialos *et al.,* 1995). Subsequent experiments demonstrated that TRAF1, TRAF2, and TRAF3 constitutively associate with LMP1 in EBV-transformed lymphoblastoid cell lines (Devergne *et al.,* 1996). Yeast two-hybrid analysis also identified A20 as a binding partner for TRAF1 and TRAF2 (Song *et al.,* 1996), and a direct interaction between TRAF1 and A20 was detected *in vivo*. An interaction between TRAF2 and A20 was not detected although overexpression of TRAF2 increased the interaction between TRAF1 and A20. This suggested that perhaps A20 interacts with TRAF1-TRAF2 heterodimers (Song *et al.,* 1996). To determine whether A20 could directly interact with LMP1, LMP1 was tagged with the FLAG epitope and A20 with a c-myc epitope to facilitate immune precipitation. H1299-p53 cells were transfected with LMP1 alone, as a negative control, or with LMP1 and A20 followed by immunoprecipitation of A20 (Fig. 1). Immunoblot analysis of the total lysate indicated that LMP1 was expressed at equal levels in both sets of transfected cells; however, it was only detected in the A20-immune complexes. The precipitation of LMP1 with A20 suggests that LMP1 specifically associates with A20.

The effect of TRAF1 and TRAF2 on the LMP1 and A20 interaction

Expression of TRAF2 has been shown to increase the association of TRAF1 with A20; therefore, the effect of expression of the TRAF molecules on the association of LMP1 with A20 was determined. H1299-p53 cells were transfected with LMP1 and A20 and either TRAF1 or TRAF2. The lysates were immunoprecipitated for A20, and LMP1 was analyzed in the immune complexes by immunoblot analysis. Overexpression of TRAF2 significantly increased the LMP1-A20 interaction (Fig. 2A). This increase in the LMP1-A20 interaction was specific for TRAF2, and overexpression of TRAF1 did not significantly or consistently affect the interaction of LMP1 and A20.

The effect of the TRAF molecules on the LMP1-A20 interaction was confirmed by LMP1 immunoprecipitation from transfected lysates. Immunoblot analysis of the individual transfections detected equal amounts of A20 in each lysate, and equal amounts of LMP1 were detected in each of the immunoprecipitated complexes (Fig. 2B).

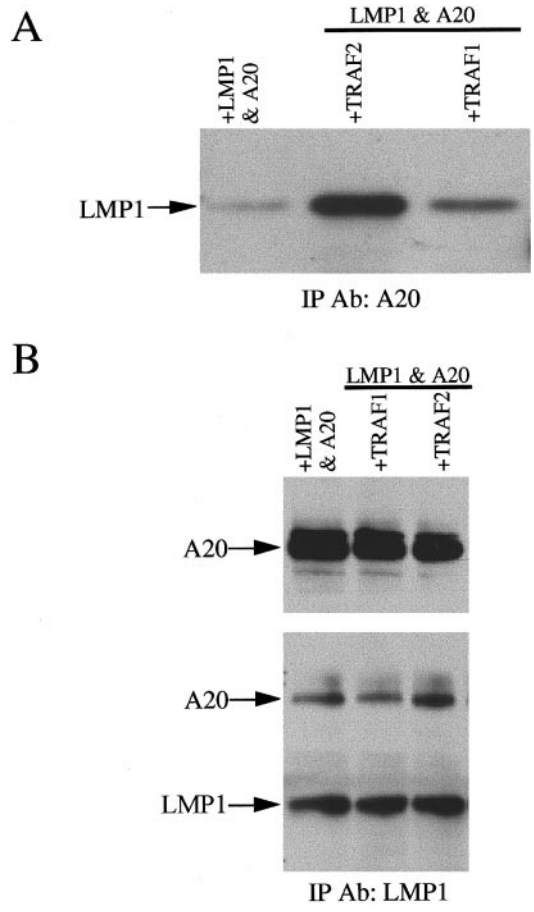

FIG. 2. Effect of TRAF1 and TRAF2 on the interaction of EBV LMP1 with A20. (A) The H1299-p53 cell line was transfected with FLAG-tagged LMP1 and A20 plus either TRAF1 or TRAF2 as indicated. The lysates were immunoprecipitated with an A20 monoclonal antibody, and the immune complexes were analyzed by immunoblot analysis with an LMP1 monoclonal antibody. (B) H1299-p53 cells were transfected with c-myc-tagged A20 and FLAG-tagged LMP1 in addition to TRAF1 or TRAF2 where indicated. Direct loads of 1% of the samples were taken before mmunoprecipitation to verify equal expression of A20. Immunoprecipitation was performed with FLAG beads, followed by immunoblot analysis with an LMP1 monoclonal antibody to determine equivalent immunoprecipitation. A20 was detected in the immune complexes with a c-myc monoclonal antibody.

Immunoblot analysis for A20 confirmed that LMP1 and A20 interact *in vivo* and that TRAF2 but not TRAF1 expression increased the interaction between LMP1 and A20 (Fig. 2B). The ability to detect the interaction of LMP1 and A20 by immunoprecipitating either protein provides strong evidence for the interaction of these proteins. Furthermore both approaches revealed that TRAF2 enhances this association.

Effect of A20 expression on the interaction of TRAF1 and TRAF2 with LMP1

To determine whether A20 binding to LMP1 affected LMP1 association with TRAF molecules, duplicate plates of H1299-p53 cells were transfected with LMP1, TRAF1, and TRAF2, either with or without A20. LMP1 immunoprecipitation was followed by immunoblotting for A20, TRAF1, TRAF2, or LMP1 (Fig. 3). Aliquots of whole cell extracts were taken before immunoprecipitation to control for equal protein expression and to determine the extent of binding. LMP1 was equally expressed in the cell lysates with and without A20, and equal amounts of LMP1 were immunoprecipitated (Fig. 3). Confirming previous studies, a significant proportion of TRAF1 was associated with LMP1. Although only trace amounts of A20 bind to LMP1, in the presence of A20 the interaction of TRAF1 with LMP1 was dramatically decreased to ~1% of the total TRAF1. In contrast, detection of TRAF2 indicated that ~2% of the TRAF2 was bound to LMP1 in the absence of A20 and that overexpression of A20 slightly decreased this association of TRAF2 with LMP1.

The effect of A20 on the association of TRAF1/TRAF2

Overexpression of TRAF2 can activate NF-kB, and TRAF1 and TRAF2 exist in complexes that are not associated with membrane-bound receptors. As previous studies have indicated that A20 binds to TRAF1/TRAF2 heterodimers, it was of interest to determine whether A20 affected the binding of TRAF1 to TRAF2. Cells were transfected with TRAF1 and TRAF2 with or without A20. Immunoprecipitation of TRAF1 followed by immunoblot analysis for TRAF2 indicated that TRAF2 is associated with TRAF1 and that A20 overexpression does not affect this association (Fig. 4).

LMP1 interferes with the A20–TRAF2 interaction

To determine whether LMP1 affected the interaction of A20 with TRAF1/TRAF2 complexes, cells were transfected with A20 and TRAF2. The association of A20 with TRAF2 then was determined in the presence or absence of LMP1. Although previous studies did not detect a direct interaction between TRAF2 and A20, in these studies, ~2% of the TRAF2 protein in the cell was detected in the A20 immune complex (Fig. 5). Equal amounts of A20 were detected in the immunoprecipitated complexes; however, the amount of TRAF2 that interacted with A20 decreased to ~0.2% in the presence of LMP1. This suggests TRAF2 has a greater affinity for LMP1 than for A20.

The effect of A20 on LMP1–TRADD and TRADD on LMP1–A20 interactions

The TRADD adaptor molecule is able to interact with the CTAR2 domain of LMP1 and is thought to mediate the activation of NF-kB by CTAR2. This CTAR2-mediated activation is decreased by inhibitors of TRAF2-mediated NF-kB activation, including TANK/I-TRAF, and by a dominant negative form of TRAF2 (Cheng and Baltimore, 1996; Kaye *et al.,* 1996; Eliopoulus *et al.,* 1997; Izumi and Kieff, 1997). As A20 also inhibits signaling from CTAR2, it was important to determine whether A20 may affect the

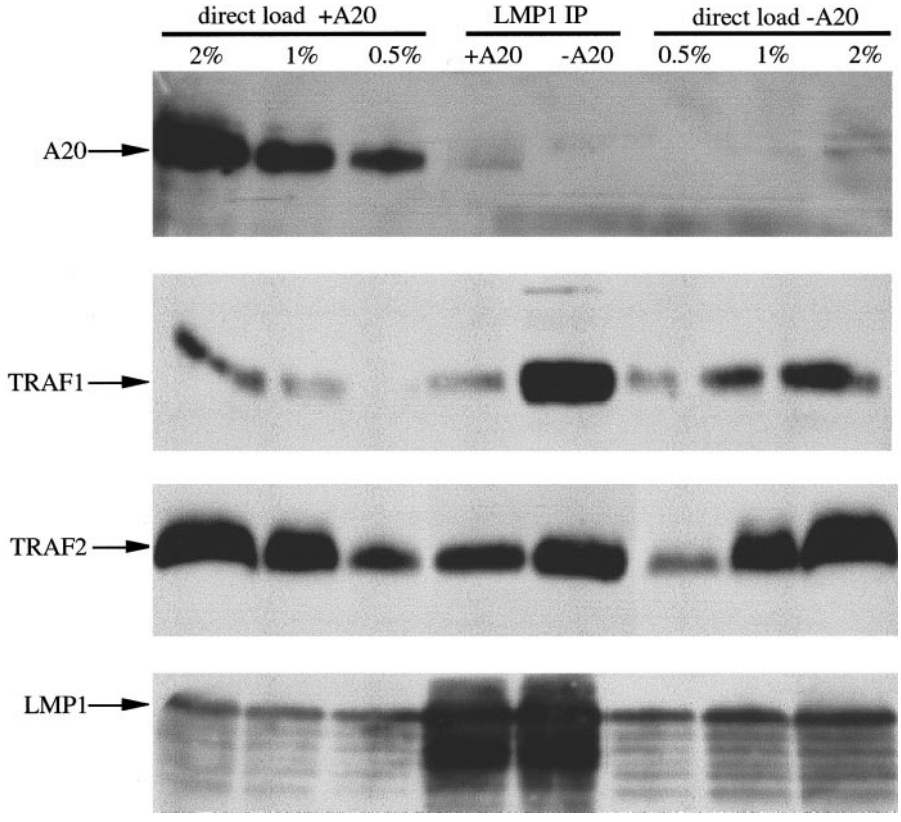

FIG. 3. Effect of A20 expression on the interaction of TRAF1 and TRAF2 with LMP1. Duplicate plates of H1299-p53 cells were transfected with FLAG-tagged LMP1, TRAF1, or TRAF2 or with the addition of c-myc-tagged A20 as indicated. The lysates were immunoprecipitated with FLAG beads and immunoblotted with the c-myc monoclonal antibody to detect c-myc-tagged A20, TRAF1 rabbit antisera, TRAF2 rabbit antisera, or the S12 LMP1 monoclonal antibody. The direct load samples were taken prior to immunoprecipitation and represent 2, 1, and 0.5% of the total lysate used for immunoprecipitations shown above.

association of LMP1 with TRADD. H1299-p53 cells were transfected with LMP1 and TRADD with or without A20. Immunoprecipitation of LMP1 followed by immunoblot analysis with a TRADD antibody confirmed that LMP1 interacts with TRADD (Fig. 6A). This interaction did not occur with a previously described mutation in the TRADD interacting domain in LMP1, 383-ID, that blocks TRADD binding. Coexpression of A20 decreased the interaction of LMP1 with TRADD although some TRADD was detected after longer exposure (data not shown).

The effect of TRADD on the LMP1–A20 interaction also was investigated. Cells were transfected with LMP1 and A20 with or without TRADD. Immunoprecipitation for LMP1 followed by immunoblotting for LMP1, A20, and TRADD detected approximately equivalent amounts of LMP1 in the immunoprecipitated complexes (Fig. 6B). A20 was readily detected in the precipitated complex, confirming the interaction of LMP1 and A20 (Fig. 6B). TRADD expression decreased but did not eliminate the binding of LMP1 and A20. These data suggest that TRADD and A20 compete for binding to the LMP1 CTAR2 domain. The residual binding of A20 to LMP1 in the presence of overexpressed TRADD most likely reflects the binding of A20 to the TRAF domain in CTAR1, which would not be affected by TRADD overexpression. The interaction of A20 with the LMP1 TRADD domain was confirmed by immunoprecipitation of A20 with LMP1 that

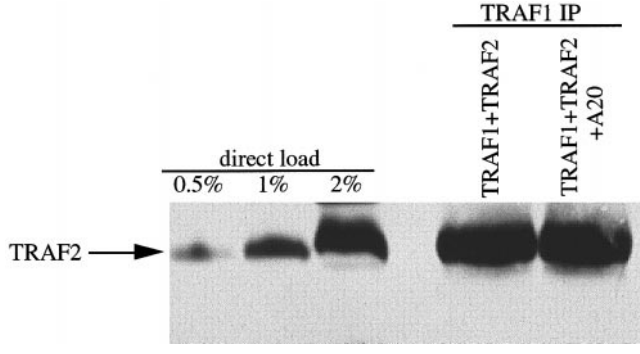

FIG. 4. Interaction of TRAF1 and TRAF2 in the presence and absence of A20. The H1299-p53 cell line was transfected with FLAG-tagged TRAF1 and TRAF2 with or without A20, as indicated. Lysates were immunoprecipitated with FLAG beads, followed by immunoblot analysis with a TRAF2 rabbit polyclonal antibody. The direct load samples were taken prior to immunoprecipitation and represent 2, 1, and 0.5% of the total lysate used for the immunoprecipitations shown above containing TRAF1 and TRAF2.

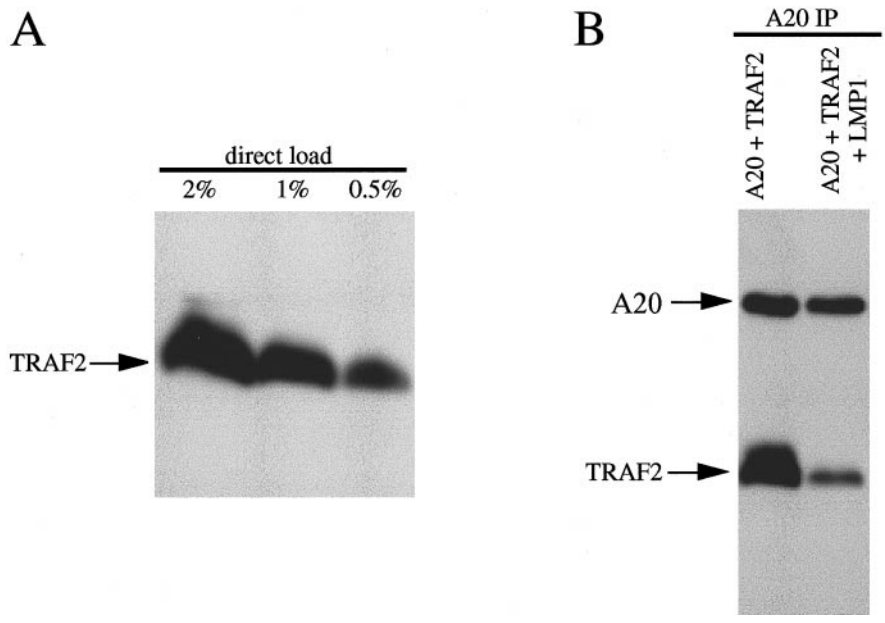

FIG. 5. Interaction of A20 and TRAF2 in the presence and absence of EBV LMP1. (A) The H1299-p53 cell line was transfected with c-myc-tagged A20, TRAF2, and FLAG-tagged LMP1 as indicated. Lysates were prepared, and before immunoprecipitation, aliquots of the whole cell lysates were taken for immunoblot analysis. The direct loads represent 2, 1, and 0.5% of the total lysate containing A20, TRAF2, and LMP1 used in (B) for immunoprecipitation. (B) H1299-p53 cells were transfected with the indicated DNAs, and immunoprecipitation analysis was performed using c-myc agarose conjugate to immunoprecipitate c-myc-tagged A20. The immune complexes were analyzed using the c-myc monoclonal antibody to verify equal amounts of A20 in the immunoprecipitated complexes and the amount of TRAF2 in the complexes was determined with TRAF2 rabbit antisera.

is deleted for the TRAF interacting motif at amino acids 204–208 (data not shown).

DISCUSSION

In this study, we present evidence that A20 and LMP1 exist in a complex *in vivo*. The interaction of A20 with LMP1 significantly affects the LMP1/TRAF complex with displacement of TRAF1 and perhaps a small amount of TRAF2 by A20. It is known that A20 blocks LMP1 CTAR1-mediated NF-kB activation, and this effect is likely caused by the displacement of TRAF1 from LMP1 by A20. The displacement of TRAF1 from the LMP1–TRAF complex by A20 would block the ability of TRAF1 to coactivate TRAF2-mediated NF-kB activation by the CTAR1 domain of LMP1 (Devergne *et al.,* 1996). Similarly, A20 blocks the activation of both NF-kB and JNK by CTAR2 (Eliopoulos *et al.,* 1999). The activation of NF-kB and JNK by CTAR2 is mediated by the adaptor molecule TRADD, and the data presented here indicate that both A20 and TRADD can interact with LMP1 and may compete for binding. This negative regulation of LMP1 signaling from both CTAR1 and CTAR2 by A20 may be an important feedback loop to modulate the levels of activated NF-kB and JNK in the infected cell.

Although A20 and TRADD seem to directly compete for binding to LMP1, the effects of A20 on TRAF1 binding are less direct and are modulated by TRAF2. Only ~2% of the TRAF2 is associated with LMP1 and 2% of TRAF2 with A20. This excess of TRAF2 would predict that the expression of LMP1 or A20 would not affect the association of TRAF2 with other molecules, and A20 did not affect the association of TRAF1 with TRAF2. In contrast, although TRAF2 increased the binding of A20 to LMP1, LMP1 dramatically decreased the A20–TRAF2 interaction. This suggests that LMP1 may interact with TRAF2/A20 complexes. TRAF2 apparently has a greater affinity for direct binding to LMP1 than to A20 such that in the presence of LMP1, A20 is displaced from TRAF2 but now binds directly to LMP1, displacing TRAF1. Importantly, these effects of A20 on the constituents of the LMP1/TRAF/TRADD complexes suggest that at least two different forms of LMP1 complexes exist: one that contains TRAF1 and TRADD in addition to other TRAFs and a second that contains A20 and TRAFs (Fig. 7). Interestingly, LMP1 induces the expression of both TRAF1 and A20 (Laherty *et al.,* 1992; Devergne *et al.,* 1998). A20 is induced independently by either CTAR1 or CTAR2 of LMP1 through the NF-kB transcription factor. In contrast, induction of TRAF1 expression, similarly to LMP1-induced expression of the EGFR, is mediated only through CTAR1 and is dependent on the direct interaction of the TRAF molecules with the CTAR1 domain of LMP1 (Devergne *et al.,* 1998; Miller *et al.,* 1998). The TRAF1/TRAF2-containing LMP1 complexes would activate NF-kB and induce expression of A20 (Fig. 7). A20 would bind to LMP1, displace TRAF1 and TRADD, and decrease the levels of activated NF-kB in the cell. A20 could also directly bind to cytoplasmic TRAF1/TRAF2 complexes and inhibit their

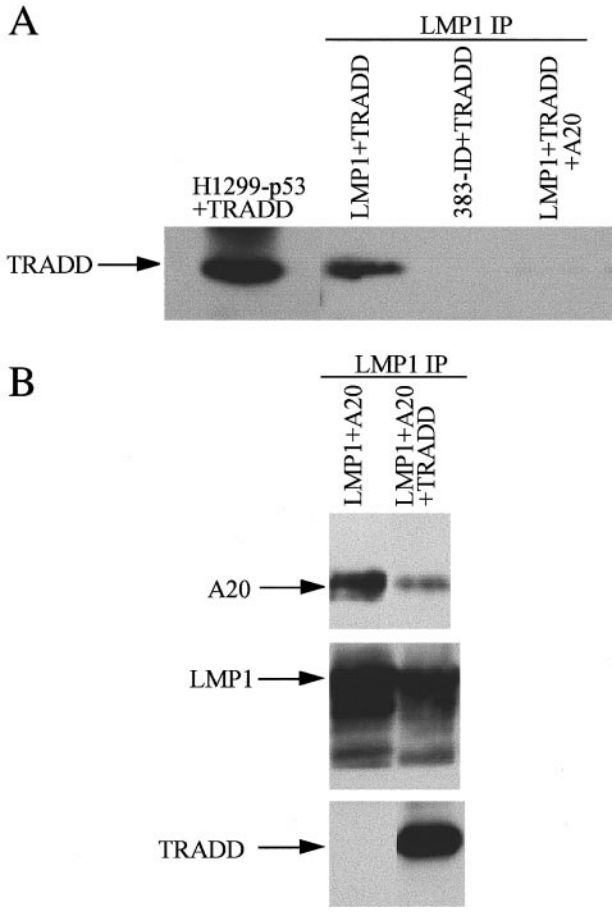

FIG. 6. The effect of A20 and TRADD on their interactions with EBV LMP1. (A) H1299-p53 cells were transfected with FLAG-tagged LMP1, TRADD, FLAG-tagged LMP1 mutant 383-ID, or c-myc-tagged A20 where indicated. Lysates then were immunoprecipitated with FLAG beads to immunoprecipitate FLAG-tagged LMP1 or FLAG-tagged 383-ID. The amount of TRADD in the immune complexes was determined by immunoblot analysis with a TRADD goat polyclonal antibody. Lysate from H1299-p53 cells transfected with TRADD was included as a positive control. (B) The H1299-p53 cell line was transfected with FLAG-tagged LMP1 and c-myc-tagged A20, with or without TRADD as indicated. Immunoprecipitation of FLAG-tagged LMP1 with FLAG beads was followed by immunoblotting with an LMP1 monoclonal antibody to verify equivalent immunoprecipitation and the c-myc monoclonal antibody to determine the amount of A20 in the immune complexes. TRADD was detected with a goat polyclonal antibody in the cells transfected with the TRADD construct.

activity. In the absence of activated NF-kB, A20 expression would decrease, enabling TRAF1 and TRADD to again bind to LMP1.

It is also possible that the A20-containing LMP1 complex may positively activate cellular gene expression and could induce expression of other genes, such as TRAF1. The distinct LMP1 complexes may coexist, perhaps in different cellular compartments, or be temporally regulated. The binding of TRAF1 or A20 could also differentially regulate signaling from CTAR1 and CTAR2. LMP1, through induction of either A20 or TRAF1, could carefully regulate the level of kB signaling and also modulate other TRAF-mediated signaling pathways.

MATERIALS AND METHODS

Cell lines

The H1299-p53 cell line is a derivative of the human non-small-cell lung carcinoma cell line stably expressing a mouse ts-p53 (Fries *et al.,* 1996). The cells were maintained at 39°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and 600 μg of G418 per milliliter (Gibco). CB5 is a human LCL established by infection of cord blood lymphocytes with B95–8 virus, and BJAB is an EBV-negative high-grade B-cell lymphoma. Lymphoid cells were maintained in RPMI 1640 medium containing penicillin and streptomycin, and supplemented with 10% fetal bovine serum.

Plasmids and expression vectors

The FLAG-tagged LMP1 expression vector contains the LMP1 cDNA, with an amino-terminal FLAG sequence, cloned into the pcDNA3 vector (Invitrogen) as previously described (Miller *et al.,* 1997). The FLAG-tagged LMP1 383-ID mutant was constructed by polymerase chain reaction (PCR), as described (Izumi and Kieff, 1997), and cloned into pcDNA3 (Invitrogen). The A20 cDNA was cloned into the *Eco*RI and *Xho*I restriction sites of the pcDNA3 expression vector. The c-myc-tagged A20 expression vector was constructed by PCR of the *Bcl*I/*Xho*I fragment of A20, using the 5′ primer (5′-ATCACGAGGCCCCCGCCTGTGATCATTTTGGCAAT-3′) and the 3′ primer (5′-ATCACGAGCATGGTGGCTC-GAGCGCCATACATCTGCTT-3′), to eliminate the A20 stop codon. The PCR fragment was ligated to the A20 *Eco*RI/*Bcl*I fragment and subcloned into the *Eco*RI and *Xho*I restriction sites of the pA3M expression vector (a derivative of pcDNA3, kindly provided by Erle Robertson), positioning the A20 cDNA in frame with three copies of the c-myc epitope at the carboxyl-terminus (Aster *et al.,* 1997). The A20 sequence was confirmed by DNA sequencing at the UNC-CH automated DNA sequencing facility on a model 377 DNA sequencer (Perkin–Elmer, Applied Biosystems Division) using the ABI PRISMDye terminator cycle sequencing ready reaction kit with AmpliTaq DNA Polymerase (Perkin–Elmer, Applied Biosystems Division). FLAG-TRAF1, TRAF1, TRAF2, and TRAF3 are all subcloned into the pSG5 (Stratagene) expression vector and were kindly provided by Elliott Kieff. The TRADD cDNA, kindly provided by David Goeddel (Tularik), is under control of the CMV promoter in the pcDNA3 expression vector (Invitrogen).

Immunoprecipitations

Transient transfections were performed using the lipofectin reagent (Gibco) according to the manufacturer's

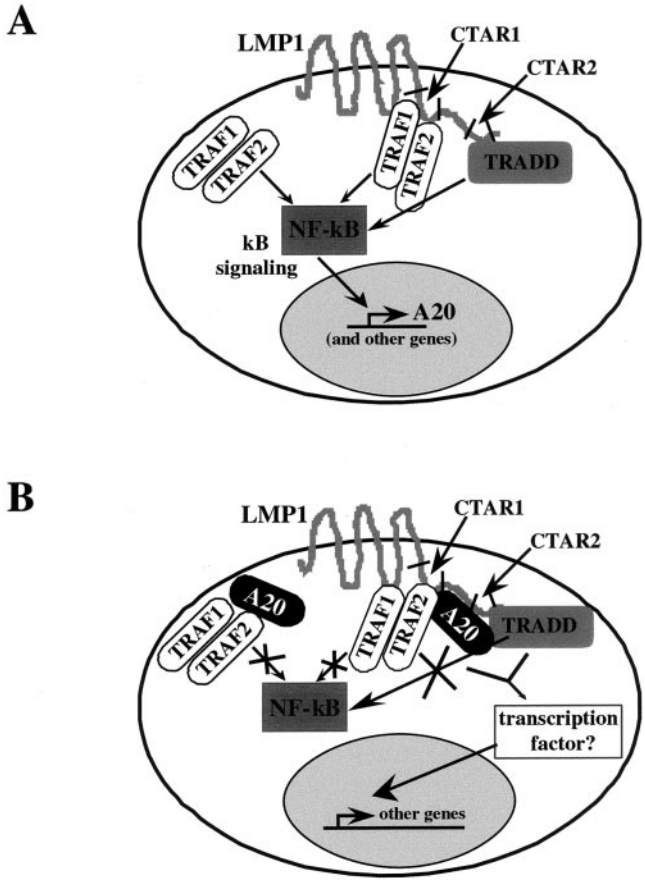

FIG. 7. Effect of A20 on the EBV LMP1 signaling complex. (A) A cartoon of EBV LMP1 located in the cell membrane, with the CTAR1 domain interacting with TRAF1 and TRAF2, and the CTAR2 domain interacting with TRADD. Both CTAR1 and CTAR2 are able to activate NF-kB, which results in the transcription of many genes, including A20. TRAF2 overexpression also activates NF-kB, indicated by the cytoplasmic TRAF1/TRAF2 complex. (B) The A20 protein is able to interact with TRAF1, TRAF2, and LMP1. The addition of A20 to the LMP1-TRAF complex significantly decreases the ability of both TRAF1 and TRADD to interact with LMP1, blocking the continuous activation of NF-kB and JNK by LMP1. A20 also can block NF-kB activation by cytoplasmic TRAF complexes. The presence of A20 in the LMP1/TRAF/TRADD complex also may result in a positive signal to an unidentified transcription factor that could activate expression of other genes.

specifications as previously reported (Fries *et al.,* 1997). Cell extracts were prepared 48 h after transfection, by incubation at 4°C for 30 min, and then scraping confluent monolayers into 1 ml of lysis buffer (20 mM HEPES, 0.5% N-P40, 250 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF, 2.5 μg/ml Aprotinin, and 25 μg/ml Leupeptin). The whole cell lysates were subjected to centrifugation, and 20 μl of anti-FLAG M2 affinity gel (Sigma) or 20 μl of anti-c-myc (9E10) agarose conjugate (Santa Cruz) was added to the supernatants. Samples were nutated overnight at 4°C, washed five times with lysis buffer, immune complexes were resuspended in 3× sample buffer, and boiled for 10 min.

A20 immunoprecipitations were performed by preclearing the supernatants with 30 μl of GammaBind G Sepharose (Pharmacia Biotech) nutating for 1 h at 4°C, adding 5 μl of A20-specific monoclonal antibody, (a gift from Vishva Dixit), (Jaattela *et al.,* 1996) to the precleared supernatant, and nutating for 2 h at 4°C. GammaBind G Sepharose was added, samples were nutated for an additional 1 h at 4°C, washed five times with lysis buffer, resuspended in 3× sample buffer, and boiled for 10 min.

Immunoblot analysis

Immune complexes were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and then subjected to immunoblot analysis. Polyacrylamide gels were transferred to a supported nitrocellulose filter (Schleicher and Schuell) with a Hoefer semidry transfer apparatus. Filters were stained with Ponceau S (Sigma) to verify equal transfer and blocked overnight in Tris-buffered saline with 0.1% Tween 20 and 5% nonfat dried milk (BLOTTO). The TRAF1, TRAF2, TRADD, and c-myc-epitope tagged A20 proteins were detected with the TRAF1 (S-19) or TRAF2 (C-20) rabbit polyclonal antibodies, the TRADD (C-20) goat polyclonal antibody, or the c-myc (9E10) mouse monoclonal, respectively (Santa Cruz), at a 1:200 dilution. The LMP1 protein was detected as described previously, using the S12 monoclonal antibody at a 1:10 dilution (Miller *et al.,* 1995). The wild-type A20 protein was detected with an A20-specific monoclonal antibody at a 1:500 dilution. Reactivity was detected using the appropriate species specific horseradish peroxidase-conjugated secondary antibodies to mouse or rabbit (Amersham), or to goat (DAKO), at a 1:1000 dilution and developed with the enhanced chemiluminescence reagents according to manufacturer's specifications (Amersham).

ACKNOWLEDGMENTS

We thank Vishva Dixit for the A20 monoclonal antibody, David Goeddel for the TRADD expression vector, and Erle Robertson for the pA3M expression vector. This work was supported by National Institutes of Health Grants CA-32979, CA-19014, and DE-11644 to N.R.T.

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